The development of an enzyme-linked immunosorbent assay for *Trypanosoma vivax* antibodies and its use in epidemiological surveys

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There are data indicating that the distribution of Trypanosoma vivax *in the Brazilian territory is expanding with potential to reach other areas, where the vectors are present. The detection of anti-trypanosomal antibodies in serum provides important information of the trypanosomal status in cattle herds. For this reason, an enzyme-linked immunosorbent assay (Tv-ELISA-Ab) with crude antigen from one Brazilian isolate of* T. vivax *was developed and evaluated. The sensitivity and specificity were respectively 97.6 and 96.9%. In the evaluation of cross-reactions, three calves inoculated with* T. evansi *trypimastigotes blood forms showed optical densities (OD) under the cut-off during the whole experimental period, except one at 45 days post-inoculation. With relation to* Babesia bovis, B. bigemina, *and* Anaplasma marginale*, which are endemic hemoparasites in the studied area, the cross-reactions were shown to be 5.7, 5.3, and 1.1%, respectively. The first serological survey of Pantanal and state of Pará showed that* T. vivax *is widespread, although regions within both areas had significantly different prevalences. Therefore, this Tv-ELISA-Ab may be a more appropriate test for epidemiological studies in developing countries because the diagnostic laboratories in most countries may be able to perform an ELISA, which is not true for polymerase chain reaction.*

Key words: *Trypanosoma vivax* - enzyme-linked immunosorbent assay - epidemiological survey - cattle - Pantanal - Brazil

Trypanosoma vivax is a haemoprotozoan found in Africa, Central and South America. In Africa, the transmission occurs cyclically and mechanically but, in the New World (America) *T. vivax* is transmitted only mechanically by tabanids and other biting flies (Dwinger & Hall 2000).

T. vivax was first reported in Brazil in 1946 parasitizing bovine in the state of Pará (Boulhosa 1946). Later, many other researchers reported the presence of *T. vivax* causing disease in bovines, ovines, caprines, and buffaloes in the same state (Shaw & Lainson 1972, Didonet-Láu 1988).

The presence of this haemoprotozoan was reported only in this region until 1996, when it was diagnosed in cattle of Pantanal region of the state of Mato Grosso (Silva et al. 1996). One year later, *T. vivax* was found infecting beef cattle in the Pantanal region of Mato Grosso do Sul (Paiva et al. 1997). These data suggest that *T. vivax* is expanding its distribution in the Brazilian territory, with potential to reach other areas, where vectors (possibly tabanids) are present.

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As in other countries of South America, the trypanosomiasis outbreaks in cattle described in Pantanal were severe, since the herds showed high morbidity with fever, lethargy, anemia, loss of physical conditions, abortion, and mortality (Losos & Ikede 1972, Dávila et al. 2003). According to Seidl et al. (1999), the economic impact due to *T. vivax* infection could exceed US$ 160 million to the cattle industry in the Brazilian Pantanal and Bolivian lowlands. However, outbreaks with these characteristics are sporadic in Latin America countries where *T. vivax* occurs endemically, but the infection of this flagellate still being considered economically relevant (Otte et al. 1994).

One of the major parameters considered in modeling trypanosomosis control measures is the accurate assessment of the prevalence and/or incidence of the infection (Snow & Rawlings 1999). The parasitological techniques are the most common for *T. vivax* diagnostic in Latin America as well as in Brazil. However, in infected animals the parasitemia fluctuates greatly and low number of parasites are present in the bloodstream during the chronic phase of the infection and often below the detectable by the usual parasitological diagnostic techniques (Mattioli & Faye 1996). The enzyme-linked immunosorbent assay (ELISA) that detects *T. vivax* antigen, initially described as more sensitive than Woo test (Masake et al. 1995), showed inferior field performance (Eisler et al. 1998). The polymerase chain reaction (PCR) had considerably improved the sensitivity of *T. vivax* diagnosis (Masake et al. 1997, Ventura et al. 2001), consequently showing ad-

vantages over the parasitological diagnosis (Magona et al. 2003). However, the detection of anti-trypanosomal antibodies in serum provides accurate information on the trypanosomal status in cattle herds through prevalence determination (Connor & Halliwell 1987, Mattioli et al. 2001).

The ELISA for detection of antibodies against trypanosome are often based on crude antigens which have shown instability and cross reactions between species of trypanosomes (Luckins 1977, Greiner et al. 1997). Recently, Rebeski et al. (2000b) concluded that plates precoated with crude antigen, air dried and stored over 4ºC provided better performance than plates freshly coated with antigens kept frozen. However, Lejon et al. (2003) detected that the ELISA for *T. vivax* antibody detection in goats failed in consistency to detect the infected animals.

This paper firstly describes the development and evaluation of an ELISA with crude antigen of a Brazilian isolate of *T. vivax*. Secondly, reports seroepidemiological surveys in cattle of the Pantanal region in the state of Mato Grosso do Sul and in various micro regions in the state of Pará, Brazil.

MATERIALS AND METHODS

T. vivax isolate and parasitized blood - *T. vivax* was obtained from a naturally infected cow of the state of Mato Grosso, Pantanal wetlands, Brazil (GCTvPPO1-CO isolate). The stabilates produced with the infected blood of this cow were cryopreserved with 10% glycerol in liquid nitrogen. Two clinically healthy Nellore calves (12 months old) and negative for *T. vivax* determined by successive Woo parasitological exams (Woo 1969) were inoculated subcutaneously with the previously mentioned stabilate. Blood from these calves for the antigen preparation was collected in sodium heparin (10 UI/ml of blood) at parasitemia of approximately 40 trypomastigotes per microscopic field (1000 × magnification).

T. vivax purification and antigen preparation - Two hundred milliliters of blood with the parasite concentration mentioned above were employed in each of the three procedures for the *T. vivax* purification. The first one was through centrifugation of parasitized blood at 1090 × g during 5 min. Plasma just over the buffy coat was collected and centrifuged at 12,100 × g. The second was done according to Barbosa (2000) for purification of *T. cruzi*. Shortly, the blood was centrifuged at 453 × g for 15 min. The centrifuged tubes were placed in a water bath at 37ºC for 15 min for parasite migration into the plasma. The plasma was transferred to other tubes and centrifuged at 2600 × g for 15 min. The pellet was homogenized with Dulbecco's Modified Eagle's Medium (DMEM) containing 20% of fetal bovine serum in ice bath and after that was placed again at 37ºC for 15 min for platelet agglutination. The platelet clumps were removed with a pipette aid and the remaining suspension was centrifuged at 2600 × g for 15 min to sediment the parasites. The parasites were suspended in DMEM at –70ºC until the antigen preparation. The third process was done according to Lanham and Goodfrey (1970) procedure. Shortly, DEAE-Sephadex columns (DE52, Whatman, catalog 4057050) were washed and equilibrated with phosphate saline buffer pH 8.0 (9.5 mM, Na_2HPO_4, 5 mM $NaH_2PO_4.2\,H_2O$, 72.6 mM NaCl) containing 1% glucose (PSG) in a 4:6 range. The DEAE-Sephadex slurry was placed carefully in Buchner funnels with filter paper (Whatman no. 41) on the botton. In the equilibrated and packed columns covered by filter paper (Whatman, no. 41) it was placed parasitized blood diluted 1:2 in PSG. The eluted *T. vivax* was saved in glass tubes and samples of each one were examined in the microscope to evaluate the parasite concentration. The eluted parasites were pooled and centrifuged at 1800 × g for 20 min. The *T. vivax* pellet obtained from each purification method was mixed with lyses solution (100 mM tris, 10 mM EDTA, 0.2 mM N-α-p-tosyl-L-lysil chloromethyl ketone, 2 mM phenylmethyl sulfonylfluoride and 1% Nonidet P40 v/v). This suspension was homogenized in Toen-Broeck and kept at 4ºC for 30 min and frozen at least overnight at –70ºC. After thawing, this material was again homogenized in Toen-Broeck and sonicated at 100 Watts for 4 min (Branson Sonicator model 250, Branson Ultrasonics Corporation, US). Finally, the material was centrifuged at 12,100 x g, at 4ºC for 30 min. The supernatants according the first, second, and third *T. vivax* purification procedure, were denominated as Pl-Tv, Ptf-Tv, and IEC-Tv antigens, respectively. The total protein concentrations were estimated by the Folin's reagent method and were 835 µg/ml, 643 µg/ml, and 574 µg/ml, respectively. Aliquots of 100 µl of these antigens were stored at –70ºC.

ELISA for detection of antibodies against T. vivax (Tv-ELISA-Ab) standardization - A series of experiments were performed to find the best antigen concentration, serum, conjugate dilutions, and substrate. The Pl-Tv, Ptf-Tv, and IEC-Tv antigens were diluted in such way to have the same concentration in all evaluations. It was found identical performances, therefore the first method was chosen, because it is simple, economical and less time consuming. The largest optical density difference between the mean optical density (OD) of positive (3) and negative (3) control sera was found by the protocol described below. Antigen diluted 1:1000 in Dubellccos's phosphate buffer solution (1.47 mM KH_2PO_4; 154 mM NaCl; 8.09 mM Na_2HPO_4), pH 7.3 ± 0.1 (DPBS) was added at volume of 100 µl (20 ηg/well) in each well of polystyrene 96 well plate (Kima, ref. 655101). The antigen adsorption occurred during two hours at 37ºC and subsequently, the plates were frozen overnight at –20ºC. The plates were then thawed and washed five times with phosphate buffer saline (PBS) (3.59 mM $Na_2HPO_4.2\,H_2O$; 1.23 mM $NaH_2PO_4\,H_2O$; 99.2 mM NaCl) containing 0.1% Tween-20 (PBST), pH 7.2. Then, the plates were blocked with 100 µl/well of DPBS containing 2% IgG-free equine serum (Sigma, ref. A-6917) and 5% skim milk for 4 h at 4ºC and frozen overnight at –20ºC. After thawing, the plates were washed with PBST as described above. Four replicates of a strong positive, weak positive as well as negative sera controls diluted 1:1000 in PBST were placed in adjacent wells in the volume of 100 µl of each microtitre plate. The same dilution and volume of test sera were placed into the wells in duplicate. After incubation for 45 min at 37ºC and washing as described, bound antibodies were detected with 50 µl/well of rabbit anti-bovine IgG horseradish peroxidase conjugate (Sigma, ref. A-9169), diluted 1:10,000 in PBST.

Microtitre plates were then incubated at 37ºC for 30 min and after this incubation, the plates were washed as above, dried and in each well was added 50 µl/well of o-phenyl diamine in a substrate solution. The reaction was stopped 10 min later by the addition of 100 µl of H_2SO_4 (13.3 % to each well). The results of the test were read in an ELISA reader at 490 nm filter (EL x 800, Bio-TekCorporation, US).

Cut-off establishment - The ELISA cut-off was determined with 128 cattle sera. One hundred and seven sera were from healthy cattle of the state of Rio Grande do Sul, Brazil, where *T. vivax* is not present. The remaining 21 sera were from calves raised in a diptera and tick-free isolation building at Embrapa Beef Cattle, Campo Grande, MS, Brazil. Although this area is considered *T. vivax* free, blood smears stained with May-Grunwald-Giemsa were examined before blood was collected to obtain sera. The cut-off was considered the OD mean of these sera plus three standard deviations. It was determined by plate and were accepted the plates with cut-off coefficient of variance equal or less than 5%. The OD of these plates were adjusted to reference plate cut-off according formula described elsewhere (Madruga et al. 2000).

ELISA performance evaluation - Sensitivity was determined with 85 cattle sera. Sixty five sera were from cattle of Pantanal region of Mato Grosso do Sul, naturally infected with *T. vivax* as determined by the Woo test. The other 20 sera were from calves experimentally infected with 10^7 *T. vivax* trypomastigotes.

Specificity was determined with 187 sera. One hundred and twenty five sera were of cattle raised in different periods in the isolation building and kept in the serum bank at Embrapa Beef Cattle. Additionally 62 sera from Pombal county of the state of Paraíba, considered free area for this hemoparasite, were tested.

Five Nellore 6-7 months-old calves were inoculated with 10^7 *T. vivax* trypomastigotes with the objective to determine the first detection and persistence of antibodies against *T. vivax*. During the experimental period, these animals were serologically monitored by Tv-ELISA-Ab, parasitologically through May-Grunwald Giemsa stained blood smears from inoculation (day 0) and after that every 15 days until 60 days post-inoculation.

Cross-reactions with *T. evansi* antibodies were evaluated by experimental infection in three 6-10 month-old calves with 1.7 x 10^8 trypomastigotes of this species of trypanosome. Sera from these animals were collected until 90 days post-inoculation.

Cross reactions were also evaluated with sera from calves experimentally infected with *A. marginale* (89 sera), *B. bovis* (87 sera), and *B. bigemina* (95 sera) kept in the serum bank at Embrapa Beef Cattle. The indirect immunofluorescent antibody technique against *Babesia* organisms (Araújo et al. 1998a) and the *A. marginale* (Araújo et al.1998b) detected antibodies in all sera used for these cross reactions evaluations.

Seroepidemiological survey - A survey was carried out with sera from beef cattle (predominantly Nelore breed), of both sexes and varying ages, which were randomly selected from Corumbá (548 cattle sera), Porto Murtinho (150 cattle sera), Miranda (214 cattle sera), Rio Verde (579 cattle sera), Rio Negro (501 cattle sera), and Aquidauana (516 cattle sera) counties of Pantanal region of Mato Grosso do Sul. This is a 138,000 km^2 tropical seasonal wetland located in the center of South America, including part of Mato Grosso and Mato Grosso do Sul, between 16ºS and 21ºS and 55ºW and 58ºW and contains approximately 1100 extensive cattle ranches, varying from 10,000 to 200,000 hectares and three million cattle (Seidl et al. 1999).

A serological survey was also done in the state of Pará, localized between 2ºS and 10ºS and 46ºW and 59ºW in the equatorial area characterized as a tropical humid region. One thousand and fifty six cattle sera from five different regions of Pará, Marajó Island (101 cattle sera), Belém Metropolitan area (213 cattle sera), southwest (117 cattle sera), northeast (116 cattle sera), and southeast (509 cattle sera) were examined by Tv-ELISA-Ab. Most of the surveyed animals were beef cattle.

Statistical analysis - Data were analyzed by student test and qui square using the programs InStat and Prism 2 (GraphPad Software Inc.).

RESULTS

The crude soluble antigen produced from the three purification procedures showed identical performance in the standardized ELISA. This test resulted in a cut-off of 0.433 in the standard plate, considering the average OD of negative sera 0.232 plus 3 standard deviations. In the evaluation of positive sera two out of 85 sera samples from naturally and experimentally infected cattle were negative in the ELISA (Fig. 1) which corresponds to a sensitivity of 97.6%. Six out of 187 negative sera had OD above the cut-off, giving a specificity of 96.9%. All calves displayed seroconversion after the experimental inoculation that persisted until the end of the observation, except for one calf, as can be seen on Fig. 2. All three calves inoculated with *T. evansi* trypomastigotes showed OD under the cut-off during the whole experimental period, except one at the 45th day post-inoculation (Fig. 3). The sera of cattle with *B. bovis, B. bigemina,* and *A. marginale* infection, showed 5.7, 5.3, and 1.1%, of cross-reactions, respectively, as can be seen on Fig. 4. The overall prevalence of *T. vivax* antibodies in the Pantanal of Mato Grosso do Sul and Pará can be seen in the Tables I, and II.

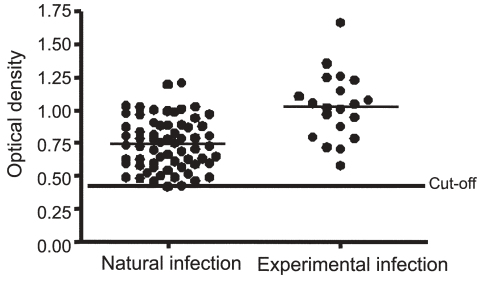

Fig 1: optical densities of the sera from parasited cattle of endemic area or experimentally infected for determination of the sensitivity by the enzyme linked immunosorbent assay with *Trypanosoma vivax* crude antigen for detection of antibodies.

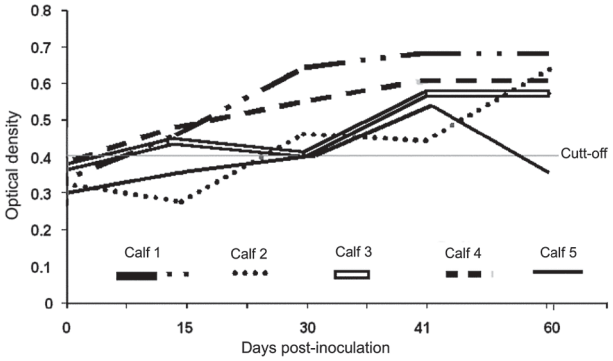

Fig 2: optical densities (490 nm) of the calves sera infected experimentally with *Trypanosoma vivax* tripamastigotes in the enzyme-linked immunosorbent assay for antibody detection.

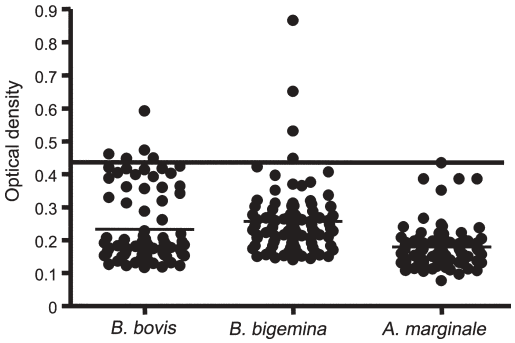

Fig. 4: optical densities of cattle sera with antibodies against *Babesia bovis, Babesia bigemina*, and *Anaplasma marginale* determined by enzyme-linked immunosorbent assay with crude antigen of *Trypanosoma vivax*.

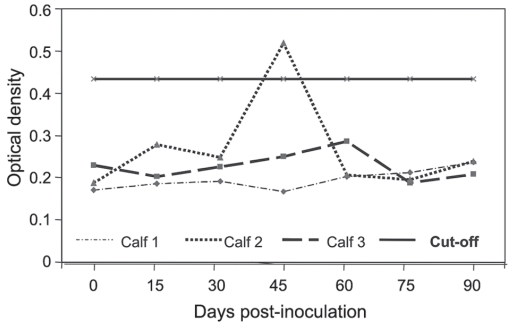

Fig 3: optical densities (490 nm) of sera from calves inoculated with 1.7×10^8 *Trypanosoma evansi* tripamastigotes in the enzyme-linked immunosorbent assay with crude antigen of *Trypanosoma vivax* for detection of antibodies.

DISCUSSION

All three *T. vivax* purification techniques yielded enough parasites for antigen production which did not differ in quality for the Tv-ELISA-Ab. For this reason the centrifugation method was chosen because plasma recovery is simpler, less time consuming and economic. The Tv-ELISA-Ab displayed a sensitivity (97.6%) which was similar to those determined by enzyme immunoassay with denatured *T. vivax* antigen (95.1%) reported by Rebeski et al. (2000a,b), and Hopkins et al. (1998) with eluate from dried blood spotted on filter paper (96.8%). However the sensitivity of Tv-ELISA-Ab was considerably higher when compared with the latter ELISA that showed sensitivity

TABLE I

Prevalence of *Trypanosoma vivax* in six counties of the Pantanal region, state of Mato Grosso do Sul, Brazil, determined by enzyme-linked immunosorbent assay with crude antigen of this haemoprotozoan for detection of antibodies

County	Cattle number	Positive sera	Prevalence (%)
Corumbá	548	394	71.9 [a]
Miranda	214	127	59.3 [b]
Aquidauana	516	359	69.6 [a]
Rio Negro	501	141	28.1 [d]
Rio Verde	579	298	51.5 [b,c]
Porto Murtinho	150	86	57.3 [b,c]
Total	2508	1405	56

a, b, c, d: statistical significance (p = 0,05) between the prevalences of each county.

TABLE II

Prevalence of *Trypanosoma vivax* in five regions of the state of Pará, Brazil, determined by enzyme-linked immunosorbent assay with crude antigen of this haemoprotozoan for detection of antibodies

Region	Cattle number	Positive sera	Prevalence (%)
Marajó Island	101	70	69.3 [a]
Belem Metropolitan	213	94	44.1 [b]
Southwest	117	36	30.8 [c]
Northeast	116	28	24.1 [c,d]
Southeast	509	96	18.9 [d]
Total	1056	324	30.7

a, b, c, d: letters indicated statistical significance (p = 0,05) between the prevalences of each county.

of 86.1%, when performed with serum only instead serum impregnated in filter paper. The 96.6% specificity of Tv-ELISA-Ab was also similar to other ELISA tests such as the ELISA with denaturated *T. congolense* antigen (94.2%) or with *T. vivax* antigen using sera or bloodspots, 95.2 and 95.7%, respectively (Hopkins et al. 1998).

Contrary to the ELISA described by Luckins (1977) for *T. vivax* antibodies in cattle, the Tv-ELISA-Ab differentiated *T. evansi* infections because only one cross reaction with sera of one of the calves experimentally infected with this species was observed. Cross reactions were expected because the crude antigens from whole trypanosomes are considered of limited subgenus specificity (Magona et al. 2003). The degree of *T. evansi* antigens involvement in the cross-reactions is not clear. On one side, at least one *T. evansi* variant surface glycoprotein (VSG), p64 induces antibody production that recognizes epitopes on the homologue *T. vivax* VSG (Uzcanga et al. 2004). On other side the lower number of variable antigen type (VAT) diversity displayed by *Trypanozoon* sub-genus, to which *T. evansi* fits in, compared to *T. vivax* could be a limitation for cross-reactions (Meirvenne 1996). Perhaps, the high serum dilution compared to other ELISA tests, may contribute to the good sensitivity showed by Tv-ELISA-Ab. Despite sera dilution, the ELISA sensitivity was not reduced, since the mean of the first detection of antibodies occurred around 15 days after inoculation which do not differ from the ELISA with denatured antigen described by Rebeski et al. (2000b). Any way, the *T. evansi* infection at least in the Pantanal will not affect the seroprevalence studies, because Franke et al. (1994) found low seroprevalence of this species of trypanosome in bovines of northern Pantanal. Also, Davila et al. (2003) found a low percentage of cattle from Nhecolandia region, southern Pantanal, infected with *T. evansi* using polymerase chain reaction in the epidemiological study.

An advantage of the use of Tv-ELISA-Ab in serological studies in Latin America is the absence of other trypanosome species that induce immune response which shows strong cross reactions in the ELISA with *T. vivax* crude antigen as occurs in Africa with *T. congolense* (Rebeski et al. 2000b). Another factor in favor of Tv-ELISA-Ab is that *T. theilleri,* which occurs in South America, does not show cross reaction in the ELISA with *T. vivax* antigen (Luckins 1977). Although cross-reactions with *B. divergens* and *A. marginale* have not been verified (Luckins 1977), the Tv-ELISA-Ab exhibited a low percentage of cross reactions with *B. bigemina, B. bovis*, and *A. marginale,* blood parasites that occur endemically in the Pantanal and other Brazilian regions (Barros et al. 2005). Probably these reactions were restricted to an acute phase of primary infections of these hemoparasites, due to the immune response of some individuals that induced production of low affinity antibodies.

The evaluation of the Tv-ELISA-Ab in epidemiological studies showed that *T. vivax* is present in the whole Pará with variable prevalences among the different microregions. This was expected because the presence of *T. vivax* in bovine, water buffalo and ovine has been reported in various locations of Pará for long time (Shaw & Lainson 1972). The highest prevalence in Marajó Island and Belém metropolitan area is possibly related to wetlands of these areas that favor the mechanical transmission by tabanids and other haematophagous insects. In the Pantanal of Mato Grosso do Sul an endemic situation for *T. vivax* was also verified. It is important to mention this, the first diagnosis of this hemoprotozoan occurred nine years ago (Paiva et al. 1997). The only previous prevalence data in Pantanal was restricted to Nhecolandia microregion, determined by polymerase chain reaction (PCR). This study included buffalo, bovine, and sheep, and a prevalence of 42% was found (Davila et al. 2003), which is distinct of the overall prevalence of 56% found by the present serological survey which included six counties of the Pantanal of Mato Grosso do Sul. Also, the prevalences the Pantanal regions were significantly distinct. However, a clear conclusion is not possible, because the sampling methodology was not appropriate for a precise epidemiological evaluation. Indeed the seasonal factor was not considered, but it may be a determinant for the prevalence. The epidemiological study in the Nhecolandia found higher prevalence in the rainy season (Davila et al. 2003). During this period, flooding occurs during 5 to 6 months, resulting in an increased vector population and a reduced area of pasture which leads to higher cattle density per area and consequently to nutritional problems. Among the various biological factors that can determine the pattern of *T. vivax* infection, the nutritional one is reported to be important for the control of this infection, either under natural (Carmichael 1948) or experimental (Schenk et al. 2001) conditions. In the dry season, the environmental conditions favor a reduced *T. vivax* inoculation rate due to a lower vector population and better infection control by the host obtained by improved nutritional conditions. Seasonal variations and the nutritional host conditions have already seem to affect the *T. vivax* prevalence. Indeed, according to Authié et al. (1993), antibodies can persist up to 4 months following self cure. On the other hand, Bocquentin et al. (1990) observed that after parasite clearing by treatment, with 55 days the ELISA OD values become negative.

Based on the results, the Tv-ELISA-Ab showed to be suitable for seroepidemiological surveys and its development with a Brazilian *T. vivax* isolate was necessary because antigenic differences of trypanosomes from different geographical areas would affect the results of bovine trypanosome serology according to Rebeski et al. (2000b).

The Tv-ELISA-Ab allowed for the first time a seroprevalence study in regions of Brazil where *T. vivax* was previously diagnosed. This serological test is a diagnostic tool that permits better prevalence studies than the parasitological tests because it has significantly higher sensitivity (Mattioli et al. 2001). In our country the Tv-ELISA-Ab may be more appropriate for epidemiological studies because most of the diagnostic laboratories can perform an ELISA test which is not true for PCR.

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